

# Neurobiochemical mechanisms of a ketogenic diet in refractory epilepsy

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A ketogenic diet is an important therapy used in the control of drug-refractory seizures. Many studies have shown that children and adolescents following ketogenic diets exhibit an over 50% reduction in seizure frequency, which is considered to be clinically relevant. These benefits are based on a diet containing high fat (approximately 90% fat) for 24 months. This dietary model was proposed in the 1920s and has produced variable clinical responses. Previous studies have shown that the mechanisms underlying seizure control involve ketone bodies, which are produced by fatty acid oxidation. Although the pathways involved in the ketogenic diet are not entirely clear, the main effects of the production of ketone bodies appear to be neurotransmitter modulation and antioxidant effects on the brain. This review highlights the impacts of the ketogenic diet on the modulation of neurotransmitters, levels of biogenic monoamines and protective antioxidant mechanisms of neurons. In addition, future perspectives are proposed.

**KEYWORDS:** Ketogenic Diet; Ketone Bodies; Refractory Epilepsy.

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## INTRODUCTION

The ketogenic diet (KD) is particularly aimed at treating children and adolescents with refractory epilepsy (drug-refractory seizures), regardless of the etiology (1). Although refractory epilepsy is the initial focus of this treatment, clinical and epidemiologic studies indicate that chronic epilepsy is followed by long-term behavioral changes and cognitive degeneration even in an optimal state of anti-epileptic drug therapy (2,3). Consequently, some authors that the KD may be an early option for the treatment of patients with epilepsy instead of the last choice. The KD is also an important adjuvant treatment for most refractory and generalized epilepsies, such as Dravet, Doose, Lennox-Gastaut and West syndromes (4).

The KD was developed in 1920 by Wilder (5) and many studies have shown its positive benefits, including an over 50% reduction in seizures, which is considered to be clinically relevant (6,7). The average time of treatment with the KD is two years, after which it should be discontinued (1).

Recently, Hirano et al. (8) reported the positive effects of the KD in children with West syndrome who were resistant to adrenocorticotrophic hormone (ACTH) therapy, which is a first-line treatment for children with this syndrome. Among the main effects observed in five out of six children in this study included the disappearance of spasms in two children and a decrease in their frequency by 80% in the other three children. Similar positive effects of the KD were observed in a study of 41 children with refractory epilepsy, in which the number of seizures was reduced by 90% in 10.53% of the children and by at least 50% in 36.84% of the children and the seizures disappeared in 5.26% of the children (9).

The KD is based on high fat, low carbohydrate and moderate protein levels and the production of ketone bodies (KBs) from the oxidation of fat as the primary source of metabolic energy, which appears to be involved in the control of seizures (10).

The modified Atkins diet (MAD) is also used in the treatment of patients with refractory epilepsy (11). As opposed to the KD, there is no restriction on protein or daily calorie intake in the MAD. This diet is composed of 60% fat, 30% protein and 10% carbohydrates (12). Although the MAD is more palatable than the KD, its efficacy in relation to the KD is unclear (11). In children with Lennox-Gastaut syndrome, the MAD was effective and well tolerated and the nine children on the diet showed an over 50% reduction in seizure frequency after one year of treatment (13). However, a previous review showed that 37% of patients who were fed the KD had an additional

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decrease ( $\geq 10\%$ ) in seizures compared with those who were fed the MAD (14).

Regardless of the use of the MAD or the KD, in some clinical situations, such as those involving patients with glucose transporter 1 deficiency syndrome (GLUT1-DS), these dietary treatments can be used as differentiating tools for identifying patients with metabolic diseases because these patients are generally seizure-free after the introduction of the diet (15).

Previously, the KD protocol recommended that the diet be initiated after a fasting period of 12-48 h, during which the child must stay at a hospital (1). As described in the references, many centers begin the diet without fasting because several studies have found no difference in the use of fasting *versus* non-fasting in clinical practice (5). The introduction of the KD following specific requirements (fat-to-carbohydrate and -protein ratios) and the subsequent control of seizures usually occur when these ratios are 3:1 or 4:1, which are the most commonly used proportions (16). Diets containing lower proportions (2:1) are normally used when the treatment is introduced (10).

The KD is usually well tolerated and increasing numbers of studies in the literature are reporting its benefits. However, the metabolic pathways involved in the production of KBs have not been well established despite nearly one century of research. This review highlights the main neurobiochemical mechanisms that have been studied over the past 15 years according to original and review studies indexed in the MedLine/PubMed database.

### Anticonvulsant mechanisms and ketone bodies

There are many hypotheses regarding the antiepileptic mechanisms of the KD. The early hypotheses regarding its activities were focused on the concepts of acidosis, dehydration and increased ketone concentrations (17). Other factors, such as  $\gamma$ -aminobutyric acid (GABA) and glutamate, membrane potentials, ion channels, biogenic monoamines and neuroprotective activities (Figure 1), have been studied in experimental models (*in vivo* or *in vitro*).

Although the mechanism by which the KD exerts its anticonvulsant effects is unclear, these effects are often associated with important metabolic changes that induce increased levels of KBs, mainly  $\beta$ -hydroxybutyrate and acetoacetate (18,19).

Energy metabolism in the brain involves distinct and complex pathways. Under physiological conditions, most precursors of KBs are long-chain fatty acids. They are released from adipose tissue in response to a decrease in blood glucose, such as that which occurs during fasting (20).

Similar mechanisms are involved in the KD, during which long-chain fatty acids are metabolized in the liver and converted into KBs. These fatty acids are oxidized in the mitochondria, producing high levels of acetyl-CoA, which cannot be oxidized in the Krebs cycle. The excess acetyl-CoA is converted to acetoacetate and subsequently to acetone and  $\beta$ -hydroxybutyrate (21). The KBs cross the blood-brain barrier and are transported by monocarboxylic acid transporters to the brain interstitial space, the glia and the neurons. In these tissues, the KBs act as substrates in the Krebs cycle and respiratory chain, contributing to brain energy metabolism (21).

Currently, there is no evidence that dehydration or fluid restriction is necessary for the clinical efficacy of the KD (17). Furthermore, this diet has been associated with pH

changes that directly influence the behaviors of ion channels and neurotransmitter receptors (17).

Some studies have suggested that the KD is more effective in children than in adults. There are high levels of ketone-metabolizing enzymes in the brain and their capacities for taking up ketone bodies are higher in infancy than in adulthood (18,21). The number of monocarboxylic acid transporters decreases with cerebral maturation and they are present at low levels in adulthood (21). Despite these differences, adaptive cerebral metabolic changes occur in adults who are exposed to stress situations, such as ischemia, trauma and sepsis (22). As shown in the literature, there are increases in the concentrations of ketone-dependent monocarboxylic acid transporters in these situations, indicating that KD treatment in adults is feasible (22,23).

Several studies of the mechanisms of action of the KD have been based on animal models, allowing for the investigators to examine the anatomical, chemical, cellular, molecular and functional changes that occur following seizures (24,25). Different animal models have been used that have been exposed to electrical and chemical stimulation and physical, genetic and spontaneous seizure models have been employed that simulate different types of epileptic seizures (17,21,26). Table 1 shows the main outcomes reported in recent years.

The *in vivo* and *in vitro* models have revealed the different anticonvulsant properties and antiepileptic effects of the KD. These aspects have been studied primarily in models of non-epileptic rodents receiving the KD that are later exposed to proconvulsant agents or electrical stimuli (18). However, the levels of therapeutic KBs and the specific effects of each ketone body have not been clearly elucidated.

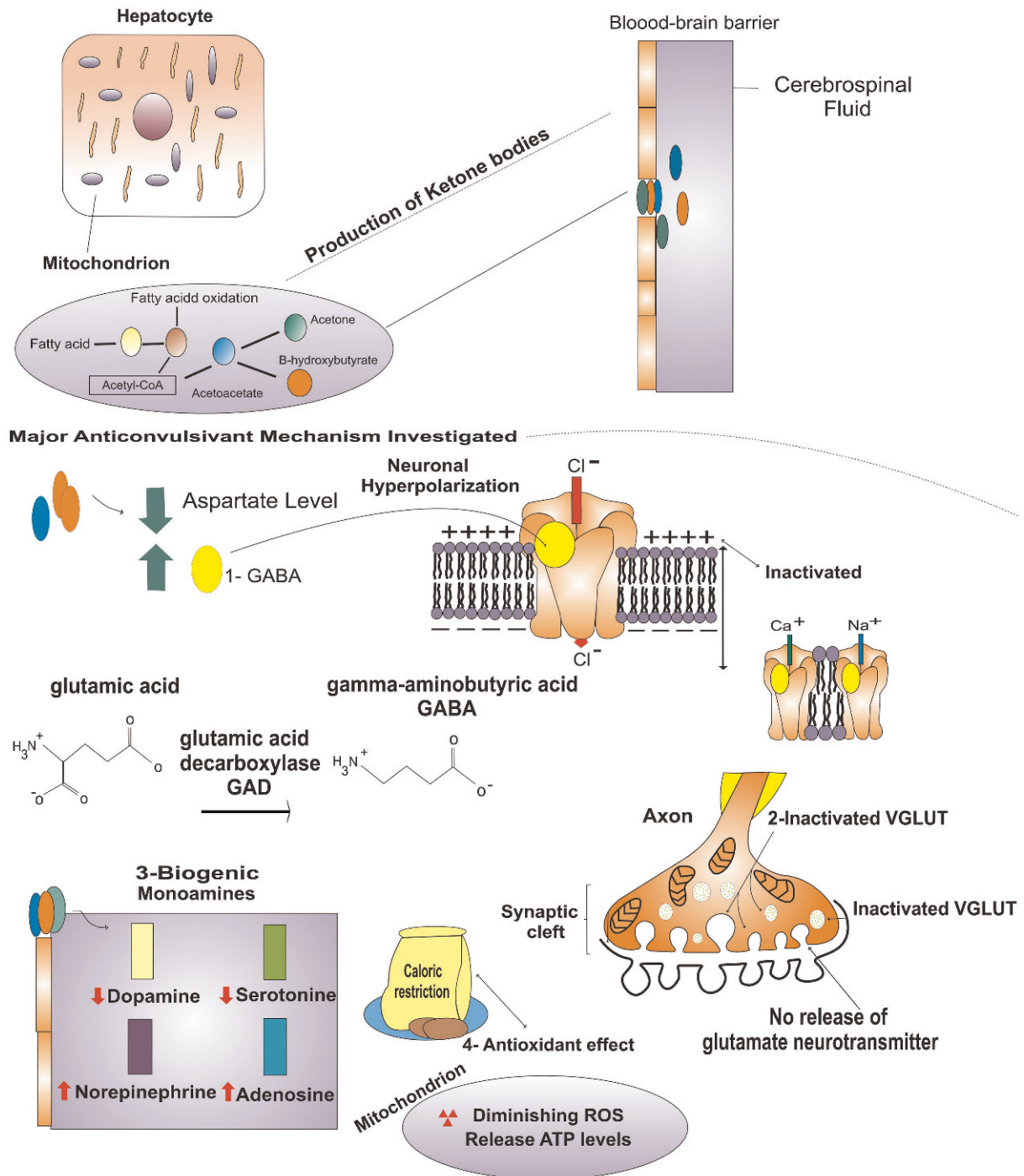
In 2003, Likhodii et al. (27) administered intraperitoneal injection of acute acetone to rats in increasing doses from 2 to 32 mmol/kg. These authors observed an increase in the protective effect of acetone against seizures as the dose increased in four different models: maximal electroshock, subcutaneous pentylenetetrazol, amygdala kindling and AY-9944 (27). Gaisor et al. (26) showed similar results following the administration of acetone (1-32 mmol/kg) to juvenile mice, which was shown to protect them from seizures induced by pentylenetetrazol and 4-aminopyridine. However, acetone doses of  $\geq 10$  mmol/kg promoted toxic effects in the pentylenetetrazol model, generating motor impairment in the mice.

### Modulation of neurotransmitters

The major mechanisms proposed to explain the increased inhibition and/or decreased excitation that are induced by the KD involve the neurotransmitters GABA and glutamate (28). KBs act not only as energy sources but also contribute to reducing glucose consumption in the brain by modulating the activities of neurotransmitters (29).

Changes in the levels of glutamate and GABA, which are the major excitatory and inhibitory neurotransmitters, respectively and their receptors have been proposed as the possible mechanisms of action of the KD (30). GABA is an intermediate of  $\alpha$ -ketoglutarate, which is synthesized in the Krebs cycle (via glutamate) and converted into GABA by glutamate decarboxylase (21). Moreover, KBs inhibit glutamate decarboxylase and decreased levels stimulate the synthesis of GABA, thus contributing to seizure control (31).

In previous experimental studies, animals were fed the KD and were observed to have higher concentrations of



**Figure 1** - Production of ketone bodies and potential primary anticonvulsant mechanisms: (1) GABA neurotransmitter (neuronal hyperpolarization and membrane channels); (2) inactivation of VGLUT and inhibition of glutamate neurotransmitter; (3) modified concentrations of biogenic monoamines; and (4) antioxidant mechanism of diminishing reactive oxygen species. For more information, please see text.

$\beta$ -hydroxybutyrate in the forebrain and cerebellum, indicating increased GABA levels (32). Astrocytes and neuroglial cells, which are also enriched with this enzyme during ketone metabolism, utilize KBs as energy sources (23,33). Suzuki et al. (33) suggested that the inhibition of GABA-transaminase mRNA expression was mainly dependent on  $\beta$ -hydroxybutyrate in astrocytes following the presence of increased GABA levels in the brain (33). This allows

glutamate to be more available for GABA synthesis, favoring the hypothesis that  $\beta$ -hydroxybutyrate leads to the inhibition of neuronal firing following recurrent neuronal activity (34).

Similar results were observed in a clinical study in which the GABA levels of responders were higher compared with those of non-responders following treatment with the KD (30). However, an evaluation of the dependence of this



**Table 1 - Neuroprotective effects of ketone bodies.**

Species	Injury models	Intervention times	Treatments	Effect on seizures	Mechanisms	References
Rats	Maximal electroshock or subcutaneous pentylenetetrazol or amygdala kindling or AY-9944	1- 6 days	Acetone injection	↓	Anticonvulsant effect of acetone <sup>a</sup>	27
Mice	Pentylenetetrazol, 4-aminopyridine	15-240 min	Acetone injection	↓	Anticonvulsant effect of acetone <sup>a</sup>	26
Mice	-	3 days	KD	Not rated	↑ GABA	32
Rats (cultured astrocytes)	-	5 days	β-hydroxybutyrate	Not rated	↓ GABA transaminase mRNA	33
Humans (children with refractory epilepsy)	-	3-6 months	KD	↓	↑ GABA	30
Rats (hippocampal slices)	Antidromic stimulation	40 min	β-hydroxybutyrate	↓	↑ K <sub>ATP</sub> channels	34
Rats	-	3 weeks	KD	↓	↑ Brain K <sub>Bs</sub> and ↓ glucose uptake	29
<i>In vitro</i> (proteoliposomes)	-	n.d.	Acetoacetate	Not rated	↓ Glutamate	36
Mice (norepinephrine transporter knockouts)	Maximal electroshock	n.d.	KD	↓	↑ Norepinephrine <sup>b</sup>	38
Humans (children with refractory epilepsy)	-	3 months	KD	↓	↓ Dopamine and serotonin	19
Mice (with adenosine deficiency)	Kainic acid	4-6 weeks	No therapy	↑	↓ Adenosine	40
Mice (transgenic models)	-	3 weeks	KD	↓	↑ A <sub>1</sub> R	41
Mice (hippocampal slices)	-	3 weeks	KD	↓	↑ Number of mitochondria	43
Mice (hippocampal mitochondria)	-	10-12 days	KD	Not rated	↑ UCP levels and ↓ ROS	44
Rats (hippocampal mitochondria)	-	3 weeks	KD	Not rated	↑ GSH and ↓ mitochondrial H <sub>2</sub> O <sub>2</sub>	45

GSH: glutathione; KD: ketogenic diet; ROS: reactive oxygen species; UCP: uncoupling protein; -: without seizure-inducing substance; n.d.: not described; <sup>a</sup>: very high doses of acetone may have contributed to motor impairment; <sup>b</sup>: norepinephrine transporter knockout mice and mice fed the KD showed similar reductions in seizure severity.



response on the levels of  $\beta$ -hydroxybutyrate was not performed.

Increased inhibition or decreased excitability, if sufficiently intense, may influence the normal functioning of the brain in addition to controlling seizures (28). Furthermore, high GABA levels appear to stimulate chloride channel receptors, increasing the influx of negatively charged ions and consequently inducing neuronal hyperpolarization (32). This event is responsible for inhibiting the activation of sodium and calcium channels, the activities of which are required for neuronal excitation. KBs possibly contribute to the activities of  $K_{ATP}$  channels, which experience activity-dependent opening and could partially explain the reduced numbers of epileptic seizures (34).

In contrast to the high levels of GABA, the glutamate-to-ketone ratio can modulate glutamate physiological functioning through VGLUT, which is responsible for filling presynaptic vesicles with glutamate in a  $Cl^-$ -dependent manner (35). An *in vitro* study showed that  $Cl^-$  is an allosteric activator of VGLUT, which is competitively inhibited by KBs (more often by acetoacetate than by  $\beta$ -hydroxybutyrate) (36).

### Biogenic monoamines

The modulation of biogenic monoamine levels was proposed as a plausible mechanism for explaining the anticonvulsant effects of the KD. However, the specific mechanisms underlying such activities remain unclear (17,19,37,38).

In animal models, norepinephrine levels have been shown to increase in rats receiving the KD (37). This beneficial effect of the KD was not observed when norepinephrine transport was inhibited, suggesting that the noradrenergic system is required for the neuroprotective effects of the KD to occur. A similar profile was observed in norepinephrine transporter knockout mice fed normal diets (38).

A clinical study on biogenic monoamines in the cerebrospinal fluid of children treated with the KD showed that their dopamine and serotonin levels were significantly reduced [from 410 to 342 and from 158 to 137 nmol/L (16.6 and 13.3% reductions), respectively] after a three-month treatment, whereas their norepinephrine levels [from 51.7 to 51.0 nmol/L (1.4% reduction)] remained unchanged (19). These authors proposed that changes in monoamine levels are also dependent upon whether children are respondent or non-respondent to the KD.

Some authors have suggested that adenosine is the major seizure inhibitory neuromodulator and that the KD exerts a regulatory role in relation to this monoamine (39). This hypothesis was reinforced by Fedele et al. (40), who used transgenic mice for adenosine A1 receptors (A1Rs) and revealed the presence of spontaneous hippocampal electrographic seizures due to the overexpression of adenosine. Recently, the positive impact of the KD was assessed in transgenic mice with or without adenosine A1Rs. In the mice with A1Rs that were fed the KD, seizures were nearly abolished after four weeks of treatment. In contrast, these effects were not observed in the mice lacking these receptors (41).

Thus, the KD increases adenosine levels. However, its efficiency in the control of seizures depends on the expression of the A1Rs (39).

### Neuroprotective mechanisms

Many studies have shown that the epileptogenic state involves complex molecular pathways in which oxidative stress and mitochondrial dysfunction may exert important roles in neuronal programmed/controlled (apoptosis) or uncontrolled/passive (necrosis) cell death (42). Thus, investigators have given particular emphasis to the modulation of the mitochondrial biogenesis of neurons by the KD and caloric restriction, highlighting the neuroprotective role of the mitochondria as the primary key to the control of apoptosis and cell death (43,44,45,46,47,48).

Mitochondria are intracellular organelles that primarily function in the production of cellular energy in the form of adenosine triphosphate (ATP). This nucleotide is produced by the mitochondrial respiratory chain through oxidative phosphorylation, which is performed by five multienzyme complexes (complexes I-V). The dysfunction of complex I may lead to decreased ATP production, which is commonly observed in neuronal diseases (42,49). In prolonged seizures, a temporary reduction in ATP levels can contribute to cell death (50).

Bought et al. (43) showed that mice that were fed the KD for at least three weeks showed a 46% increase in the hippocampal biogenesis of mitochondria compared with the control animals. In addition, these authors observed that 39 out of 42 regulated transcripts encoding mitochondrial proteins were up-regulated, implying increased ATP production and the capacity of this diet to stabilize neuronal membrane potentials (43).

The mitochondria are the major organelles that are responsible for reducing  $O_2$  to non-oxidative substances. However, when the mitochondrial respiratory chain is deregulated (the dysfunctioning of calcium homeostasis and imbalances of membrane potentials), decreased rates of ATP generation and the overproduction of reactive oxygen species (ROS) occur (49,51). In normal conditions, 1-5% of  $O_2$  in the mitochondrial electron transport chain is not reduced to  $H_2O$ ,  $CO_2$  and ATP, stimulating the generation of ROS [ $H_2O_2$ ,  $O_2^-$ , nitric oxide (NO) and peroxynitrite] (52).

Regarding coupled changes that occur during ROS production, other authors (44) have observed that rats that were fed the KD for 10-12 days showed significant increases in uncoupling protein (UCPs) levels in their hippocampal mitochondria. These responses were related to the 15% decrease in ROS levels in the hippocampi of these animals (44). Both effects were associated with mitochondrial biogenesis and the maintenance of calcium homeostasis (43,44).

The protective effects of the KD on oxidative stress have also been observed in the antioxidant system, particularly involving glutathione (GSH), which exhibits an increased capacity for peroxide detoxification within the cell (47). In juvenile rats that were fed the KD for three weeks, increased levels of mitochondrial-reduced GSH and an increased ratio of GSH to oxidized glutathione (GSSG) were observed, suggesting that the KD improves hippocampal redox statuses and protects mitochondrial DNA from oxidative stress. During seizures, these antioxidants are depleted and oxidative stress is stimulated (45).

Recently, some studies (46,48) have reviewed these mechanisms, emphasizing that the beneficial effects of the KD also involve caloric restriction. In addition to the increased levels of UCPs and the decreased production of



ROS, these authors also reported other mechanisms involved in the control of seizures, such as decreases in both insulin-like growth factor 1 (IGF-1) and the mammalian target of rapamycin (mTOR) and increases in both sirtuins and adenosine monophosphate-activated protein kinase (AMPK).

Sirtuins are deacetylases with multiple functions related to fat oxidation and increased mitochondrial size and number. The increased expression of sirtuins may be associated with the inhibition of IGF-1 after caloric restriction (46,48). In addition, the increase in AMPK is directly related to ATP production (48).

The mTOR protein kinase is involved in multiple and complex activities in the body, participating in specific mechanisms in the nervous system. Thus, it is an exciting target for new horizons in drug discovery (53). Brain abnormalities are associated with the hyperactivation of the mTOR pathway and the KD may play an important role in inhibiting this pathway, thus conferring anticonvulsant effects. However, the underlying mechanisms are still unknown and require further exploration (48,53).

It is important to recognize that seizures stimulate the production of free radicals and mitochondrial dysfunction, resulting in a chronic redox state, neuronal changes and an increased susceptibility to seizures, leading to epilepsy (42). As a result, the KD improves the stability of the mitochondrial membrane and increases the efficiency of O<sub>2</sub> consumption, stimulating the generation of ATP and minimizing the oxidative stress-induced epileptogenic state and mitochondrial dysfunction.

### Future perspectives

Considering the aforementioned studies, we have verified that the mechanisms of action of KBs, which are involved in the reduction of epileptic seizures, are distinct and complex. In addition, the major mechanisms proposed to date are based on experimental models and few clinical studies, which have small sample sizes and uncontrolled designs. Furthermore, the multiple etiologies of epilepsy represent an important limitation to the understanding of the relationships between the KD, KBs and neuronal mechanisms in the control of seizures. Thus, we propose the following: I - that physical or chemical mechanisms employed to induce seizures should follow standardized protocols; II - that the physiological levels of KBs should be more frequently considered in experimental treatments; III - that the etiologies of epilepsy are better characterized in future clinical trials; IV - that biomarkers of treatment efficacies (levels of KBs, GABA and monoamines) are evaluated; V - that the potential side effects of treatments are systematically monitored; and VI - that novel mechanisms of action of KBs are evaluated. In consideration of these proposals, positive clinical responses to the KD remain the principal goal of this treatment. Thus, given the current state of the research, we also propose that KD intervention should be included early in clinical protocols for the treatment of children and adolescents with refractory epilepsy and not only as the last therapeutic option.

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### AUTHOR CONTRIBUTIONS

Lima PA designed and coordinated the study, prepared the table, proposed the main facets of the study, was in charge of the analysis and quality control of the data, illustrated the figure and wrote the first draft of the manuscript. Damasceno NR designed and coordinated the study, prepared the table and critically reviewed the manuscript. Sampaio LP proposed the main facets of the study and revised the manuscript. All authors contributed to the interpretation of the data and approved the final version of the manuscript.

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